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First report of *Podosphaera cercidiphylli* on endangered *Cercidiphyllum japonicum* in China

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Abstract—A powdery mildew collected from the protected and endangered species *Cercidiphyllum japonicum* was identified morphologically as *Podosphaera cercidiphylli*, an identification confirmed by sequence analyses of the internal transcribed spacer (ITS) and large subunit (LSU) of nuclear ribosomal DNA (nrDNA). This is the first report of *P. cercidiphylli* in China.

Key words—*Ascomycota, Cercidiphyllaceae, Erysiphaceae,* taxonomy

Introduction

Podosphaera Kunze (*Erysiphaceae*, *Helotiales*) is a large genus with 62 species and 13 varieties worldwide, including 39 species native to Europe (Braun 1987, 1995). Thirty-six species have been reported from Japan (Nomura 1997), 16 species from Korea (Shin 2000), and 42 species (plus five varieties) from China (Tang & al. 2018). Within China, nine species and two varieties in *P.* sect. *Podosphaera* are known to infect 26 hosts (representing 10 genera and three families), while 33 species and three varieties in *P.* sect. *Sphaerotheca* are pathogens of 115 different hosts (representing 65 genera in 19 families) (Tang & al. 2018).

Cercidiphyllum japonicum Sieb. & Zucc. (*Cercidiphyllaceae*) is a plant endemic to eastern Asia, found in only a few Asian countries including China and Japan (Pan & Liu 1999). In China, *C. japonicum* is classified as an endangered species protected for its dioecism and extremely low regeneration rate, with natural populations distributed only sporadically in mixed forests of the warm temperate zone and subtropical mountains (Zeng & al. 2020). Unfortunately, plant diseases and insect pest attack threaten *C. japonicum*, with additional environmental stress. Our research revealed that both powdery mildew and mites (*Eriophyidae*) had jointly infected *C. japonicum* trees with the chasmothecia tending to obscure patches of cell hyperplasia caused by mites. This fungus was not easy to detect as the powdery white symptoms were inconspicuous. When we discovered cell hyperplasia in *C. japonicum*, we determined that this pathogen is new to China.

Materials & methods

Collection procedures & morphological examination

Samples of *Cercidiphyllum japonicum* leaves damaged by powdery mildew and mites were collected during 2018–2020 in Ankang City, southern Shaanxi Province, China. Herbarium specimens with chasmothecia were dried in plant presses and deposited in the Mycological Herbarium of Forestry College, Northwest A & F University, Yangling, Shaanxi Province, China (HMNWAFU-CF), and the Herbarium Mycologicum Academiae Sinicae, Beijing, China (HMAS).

Chasmothecia were picked carefully from the leaves, mounted in distilled water on a glass slide, and examined under an Olympus CX41 light microscope. Their colour, shape, cell wall surface, appendage position, length, apex branching, septum, shape, size, and numbers of asci and ascospores were observed and documented in detail. We checked 50 chasmothecia taken randomly from specimens and calculated averages and standard deviations using Microsoft Excel 2019.

Black-and-white images of the fungal teleomorph and mites were produced with a Nova NanoSEM 450 field emission scanning electron microscope according to Pathan & al. (2008). Partial photochromes were obtained by microphotographic focus stacking using a Lumix DMC-G8M camera fitted with Lumix 100–300 mm F4–5.6 φ67 mm and Mitutoyo M Plan Apo L 10×/0.28 ∞/0 f=200 lenses, M26 to M67 adapter, Wemacro Rail®, and Helicon® Focus 7 and Zerene Stacker® software (see https://www.wemacro.com/).

DNA extraction

Genomic DNA was extracted from the chasmothecia with BioRad Chelex-100 following published procedures (Walsh & al. 1991, Hirata & Takamatsu 1996, Bai

& al. 2014). Twenty–thirty chasmothecia were picked using a sterilized dissecting needle under a dissecting microscope and mounted onto microslides. A coverslip was used to squeeze the chasmothecia gently until broken, both slides and coverslip were rinsed gently with 50 μL 5% Chelex-100 sterilized water solution into a 1.5 mL centrifuge tube, which was placed into a 56 °C water bath for 20 min and then centrifuged at 5000 rpm for 1 min; the tube was then placed in a 100 °C water bath for 8 min, vortexed vigorously, and centrifuged for 1 min at 15000 rpm. The supernatant was transferred into another tube to be used as the template DNA.

PCR amplification

The nrDNA ITS region was amplified by nested PCR methods using ITS5 (White & al. 1990) and P3 (Kusaba & Tsuge 1995) as the first primer pair and ITS4 (White & al. 1990) and PM5 (Takamatsu & Kano 2001) as the second pair in 20 μL volumes containing 10 μL 2× CoWin Taq PCR MasterMix, 1 μL each Shanghai Sangon Biotech primer, 2 μL extracted DNA (65.9 ng/μL), and 6 μL ddH_2O. The samples were processed in a Bioer GeneAmp PCR TC-96 beginning with a 4-min initial denaturation at 95 °C, 35 cycles of 1-min denaturation at 94 °C + 1 min at 50 °C (1 min at 60 °C for the second pair of primers) + 1 min at 72 °C, and a final 10-min extension at 72 °C, with the procedure ending at 4 °C.

The nrDNA large subunit (LSU)/28S region was amplified with the primers LSU1 and LSU2 (Scholin & al. 1999) at the same volume (20 μL) and under the same amplification conditions as the previous pair of primers ITS5 and P3. The PCR products were separated by electrophoresis on a 1% agarose gel in TAE buffer and purified using the Bioteke Zymoclean™ Gel DNA Recovery Kit according to the manufacturer's instructions. The purified DNA products were amplified again as depicted above, and the PCR products were then sequenced by Shanghai Sangon Biotech Co., Ltd.

Phylogenetic analysis

Sequences were revised in BioEdit 7.2.5, BLAST searched in NCBI, and deposited in GenBank (MN960597, MN960655). Other homologous sequences from the GenBank database were downloaded for further comparisons. DNA sequences were aligned using ClustalX vers. 1.8. (http://www-igbmc.u-strasbg.fr/BioInfo/); gaps or missing data were eliminated from the datasets.

A neighbor-joining (NJ) tree inferred from ITS and LSU sequences was constructed using MEGA X (https://www.megasoftware.net/home) with the K2+G model. Maximum-parsimony (MP) and maximum-likelihood (ML) were generated with PAUP 4.0b10 (http://phylosolutions.com/paup-test/) as described by Jiang & al. (2011) and RAxMLGUI 2.0 (Edler & al. 2021) under a GTR+GAMMA model. Bootstrap percentages (BP) derived from 1000 replicates are indicated at the tree nodes.

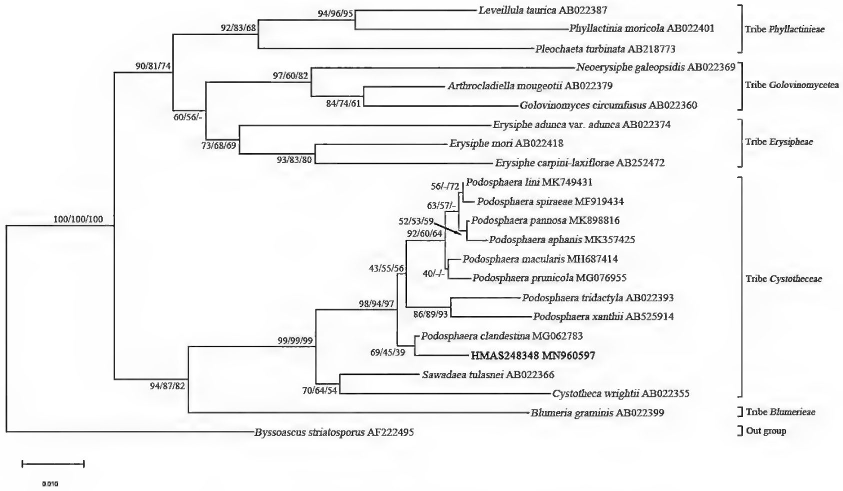
94/96/95
Leveillula taurica AB022387
92/83/68
Phyllactinia moricola AB022401
Tribe Phyllactinieae
Pleochaeta turbinata AB218773
90/81/74
Neoerysiphe galeopsidis AB022369
97/60/82
Arthrocladiella mougeotii AB022379
Tribe Golovinomycetea
84/74/61
Golovinomyces circumfusus AB022360
60/56/-
Erysiphe adunca var. adunca AB022374
73/68/69
Erysiphe mori AB022418
Tribe Erysipheae
93/83/80
Erysiphe carpini-laxiflorae AB252472
56/-/72
Podosphaera lini MK749431
63/57/-
Podosphaera spiraeae MF919434
100/100/100
52/53/59
Podosphaera pannosa MK898816
92/60/64
Podosphaera aphanis MK357425
Podosphaera macularis MH687414
43/55/56
40/-/-
Podosphaera prunicola MG076955
Tribe Cystotheceae
Podosphaera tridactyla AB022393
98/94/97
86/89/93
Podosphaera xanthii AB525914
99/99/99
Podosphaera clandestina MG062783
69/45/39
HMAS248348 MN960597
Sawadaea tulasnei AB022366
94/87/82
70/64/54
Cystotheca wrightii AB022355
Blumeria graminis AB022399
Tribe Blumerieae
Byssoascus striatosporus AF222495
Out group
0.010

Fig. 1. Topological phylogram inferred from LSU rDNA from 21 taxa of *Erysiphaceae* with *Byssoascus striatosporus* as outgroup. Bootstrap percentages from methods of neighbor-joining (NJBPs), maximum-parsimony (MPBPs), and maximum-likelihood (MLBPs) are indicated at nodes.

Results

LSU rDNA phylogenetic analyses

PCR amplification with LSU primers produced a unique 550 bp sequence. The BLAST search revealed that HMAS 248348 had a high similarity to *Podosphaera* spp. The phylogenetic tree (Fig. 1) clustered HMAS 248348 in tribe *Cystotheceae,* separating it from the other tribes in *Erysiphaceae* with strong bootstrap supports (NJBP = 99%, MPBP = 99%, MLBP = 99%). It was closely related to *P. clandestina* with a 69-scored NJBP support.

ITS nrDNA phylogenetic analyses

After amplification with the ITS5/P3 primer pair, no obvious electrophoresis products were observed due to low PCR yields. Those amplicons served as the template for the ITS4/PM5 primer pair for nested PCR, which produced a unique 450 bp sequence as checked with gel electrophoresis. That sequence was aligned with 17 GenBank sequences of tribe *Cystotheceae.* ITS nrDNA phylogenetic analyses (Fig. 2) clustered HMAS 248348 and *Podosphaera cercidiphylli* in a monophyletic clade with

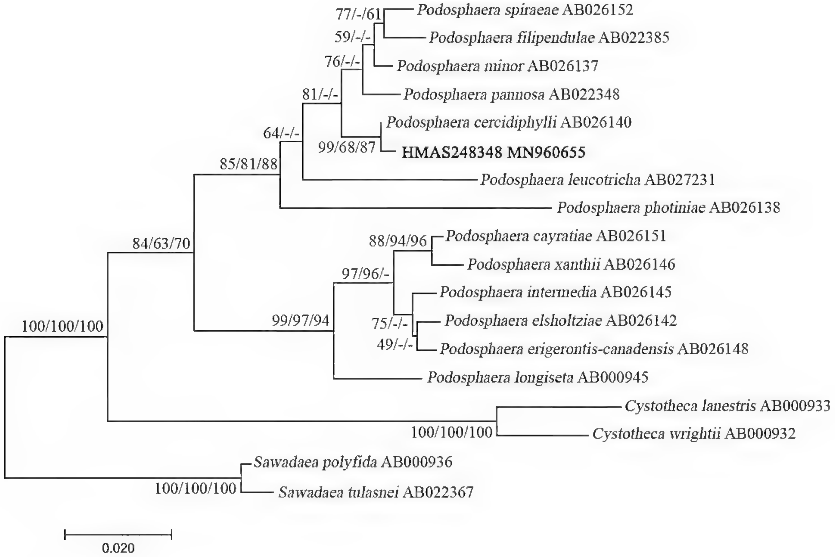
77/-/61
59/-/-
76/-/-
81/-/-
64/-/-
85/81/88
99/68/87
84/63/70
88/94/96
97/96/-
99/97/94
75/-/-
49/-/-
100/100/100
100/100/100
100/100/100
Podosphaera spiraeae AB026152
Podosphaera filipendulae AB022385
Podosphaera minor AB026137
Podosphaera pannosa AB022348
Podosphaera cercidiphylli AB026140
HMAS248348 MN960655
Podosphaera leucotricha AB027231
Podosphaera photiniae AB026138
Podosphaera cayratiae AB026151
Podosphaera xanthii AB026146
Podosphaera intermedia AB026145
Podosphaera elsholtziae AB026142
Podosphaera erigerontis-canadensis AB026148
Podosphaera longiseta AB000945
Cystotheca lanestris AB000933
Cystotheca wrightii AB000932
Sawadaea polyfida AB000936
Sawadaea tulasnei AB022367
0.020

FIG. 2. Topological phylogram inferred from ITS sequences from 17 taxa of *Erysiphaceae* tr. *Cystotheceae* with two *Sawadaea* taxa as outgroup. Bootstrap percentages from methods of neighbor-joining (NJBPs), maximum-parsimony (MPBPs), and maximum-likelihood (MLBPs) are indicated at nodes.

fairly strong bootstrap supports (NJBP = 99%, MPBP = 68%, MLBP = 87%). Therefore, we identified HMAS 248348 as *P. cercidiphylli*.

Taxonomy

Podosphaera cercidiphylli Tanda & Y. Nomura,
Trans. Mycol. Soc. Japan 27: 25. 1986. FIG. 3

Mycelia amphigenous in epidermal hyperplasia caused by mites (*Eriophyes* sp.), mostly hypophyllous, evanescent or subpersistent, powdery layer not obvious; mites aggregating on epidermal hyperplasia, massive ecdyces of melts visible in patches; chasmothecia mostly scattered or subgregarious on epidermal hyperplasia, rarely scattered epiphyllously, brown or dark brown, lighter colour incipiently, globose or subglobose, 53.8–79.0 (average 67.0 ± 7.4) µm diam., wall cells irregularly polygonal, 6.9–18.9 × 10.4–23.8 (av. 11.4 ± 3.0 × 15.7 ± 3.1) µm; appendages 2–9 (mostly 3–7, av. 5 ± 1.8), produced from the upper to equatorial part of the chasmothecia, radial, straight or arcuately curved, thick-walled throughout, 1–3 times longer than the chasmothecial diam., 70.9–226.0 (av. 129.2 ± 40.8) µm long, 5.3–9.3 (av. 7.1 ± 0.9) µm wide at the slightly thicker base, 3.3–7.2 (av. 5.3 ± 0.9) µm wide at the upper part, 1–6(–7)-septate, 2–5 times dichotomously branching at the apex, coloured dark brown from below the branch downward, ultimate branches short and obtuse, recurved or rarely straight; ascus single, subglobose to broadly ovate, sessile, double-walled, 41.3–62.6 × 49.6–69.1 (av. 53.5 ± 4.9 × 60.7 ± 4.9) µm; ascospores 8, oblong or ellipsoidal, 8.9–15.4 × 14.2–24.9 (av. 12.6 ± 1.9 × 19.1 ± 2.7) µm.

Specimens examined: **CHINA. Shaanxi Province: Ankang city**, Zhenping County, 32.1°N 109.2°E, alt. 1024 m, on living leaves of *Cercidiphyllum japonicum*, July 2018, Zhong-dong Yu (HMNWAFU-CF 2018017); July 2019, Zhong-dong Yu & Zi-jia Peng (HMAS 248348, GenBank MN960597, MN960655); Ningshan County, 33.4 °N 108.5°E, alt. 1649 m, on living leaves of *C. japonicum*, September 2020, Zhong-dong Yu & Zi-jia Peng (HMNWAFU-CF 2020021)

Distribution: Ankang city, Shaanxi Province, China; Hokkaido and Niigata Prefectures, Japan.

Discussion

Cercidiphyllum japonicum is distributed in China and Japan, and *Podosphaera cercidiphylli* (*P.* sect. *Podosphaera*) was originally described from Japan (Tanda & Nomura 1986). Here, we provide the first report from China based on morphological and phylogenetic characters; no anamorphic forms (neither mycelial layer nor conidia) were found. Tanda & Nomura (1986) cited 1–5-septate appendages arising from the basal region of the perithecium, but our Chinese specimens usually developed 1–7-septate appendages from the upper half of the equatorial region of chasmothecia, with each appendage dichotomously branched at a right or obtuse angle, and with each branch slightly shorter than the older one. However, Tanda & Nomura (1986) showed appendages dichotomously developing at a right angle with nearly an identical length for the consecutive branches. We found that there were usually eight ascospores in a single ascus instead of seven to eight ascospores as reported by Tanda & Nomura (1986). We believe that these differences are attributable to variable intraspecific development, possibly resulting from different observation methods and weather conditions at sampling sites.

Additionally, we are the first to observe a co-infection of *Cercidiphyllum japonicum* by *Podosphaera cercidiphylli* and mites. Chasmothecia were

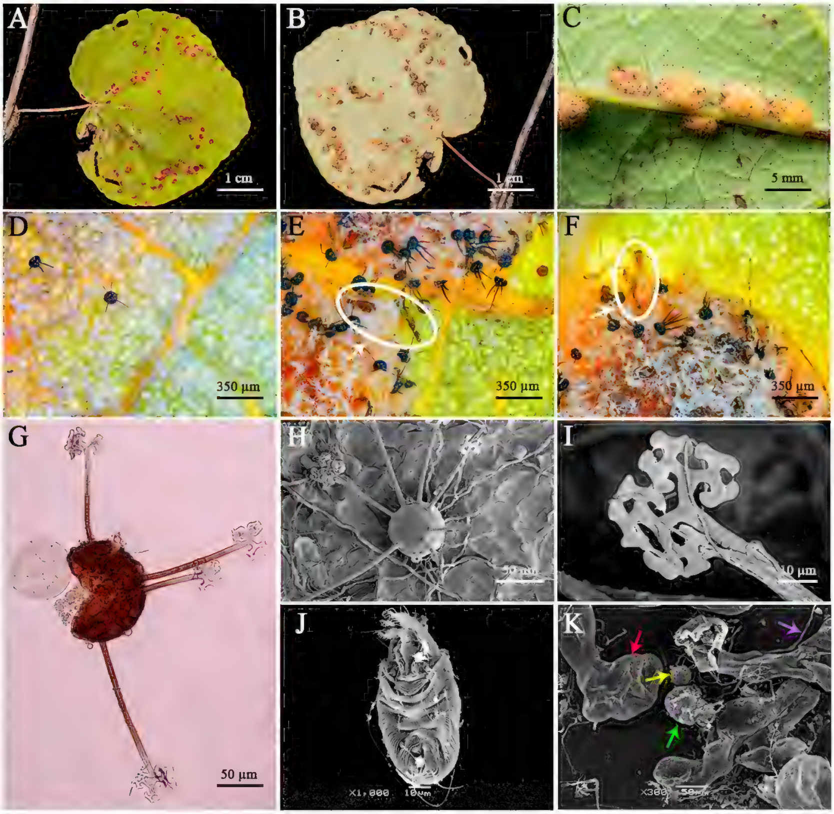

Fig. 3. *Podosphaera cercidiphylli* (HMAS 248348), teleomorphic characteristics and mites in the curled leaf: A–C. Symptoms of powdery mildew on leaf of *Cercidiphyllum japonicum*; D. Mycelia and chasmothecia; E, F. Ecdyces of mites (white arrow) and chasmothecia; G. Ascus, ascospores, and appendages; H. Insertion position of appendages; I. Dichotomous branch of appendages; J. Mite; K. Hyperplastic epidermal cells of leaf (red arrow), mycelia (purple arrow), mite (green arrow) and young chasmothecia (yellow arrow). Scale bars: A, B = 1 cm; C = 5 mm; D–F = 350 µm; G, H, K = 50 µm; I, J = 10 µm.

mainly distributed on the abaxial surface of leaves, most of which obscure patches of cell hyperplasia caused by mites. A *Podosphaera* species associated with mites was reported by Zheng & Yu (1987) occurring on *Betula pendula* Roth and *B. tianschanica* Rupr. infected by *P. erineophila* Naumov (Jaczewski 1927), but the intimate correlation between this powdery mildew and mites is not clear. We did not find the anamorphic stage of *P. cercidiphylli* on our specimens (in contrast to the Japanese specimens of Tanda & Nomura

1986); we speculate that either conidia had not been produced or that mite grazing had completely removed them. This is the first report of *Podosphaera cercidiphylli* in China.

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